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Free Progestin Levels in the Blood Plasma of Hamsters During the Estrous Cycle.

William Warren Norris Jr
Louisiana State University and Agricultural & Mechanical College

FREE PROGESTIN LEVELS IN THE BLOOD PLASMA OF HAMSTERS DURING THE ESTROUS CYCLE

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William Warren Norris, Jr.
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ABSTRACT

Levels of free plasma progestin during various phases of the estrous cycle in the golden hamster were determined using the Hooker-Forbes bio-assay and mice of the Swiss-albino strain (Rockland Farms) confirmed to be sensitive to 0.0002 µg. of progesterone. A total of 88 normal cyclic hamsters served as blood donors during estrus, early diestrus, mid-diestrus and late diestrus. Phases of the cycle were identified from vaginal smears; the estrual condition was confirmed by observation of actual copulation. Five hundred and forty uterine segments were utilized in assaying the plasma and extracts of plasma in oil samples.

A concentration of as much as 0.3 to 1.2 µg. of progestin per cc. of blood plasma was detected at pre-ovulatory estrus. During the 24 post-estrual hours this level dropped to 0.03 µg. of progestin per cc. of plasma, which level was maintained during mid-diestrus and late diestrus. There appears to be no significant rise or fall during the diestrual phases of the cycle. If the corpus luteum of the diestrual interval is functional, it is certainly functional at a low level of activity. The source of the progestin during pre-ovulatory estrus as well as during the diestrual phases requires further investigation. The adrenals cannot be excluded as a possible source.

INTRODUCTION

Perhaps the first substantial evidence that the ovary produces an internal secretion which governs the phenomenon of estrus should be attributed to Knauer (1900). Although atrophy of the uterus following removal of the ovaries had been observed much earlier, proof that the effect is mediated by the blood stream rather than via the nervous system lay in the experiments of that investigator, who showed that the uterus and secondary sex characteristics remain unaltered following autotransplantation of the ovaries.

Studies by Doisy, et al., (1924) demonstrated the presence of a hormone in the ovarian follicles of swine, cattle and women. These workers showed that a series of three subcutaneous injections of extracts of ovaries, follicles, or fresh liquor folliculi induced full estrus growth in the tissues of the genital tract of the rat. Allen and Doisy (1924) likewise demonstrated the presence of a hormone in the liquor folliculi of the hog by inducing precocious sexual maturity in immature female rats following injection of extracts of the liquor. A series of investigations by Corner (1928) and by Corner and Allen (1929, 1930) established beyond doubt that, in the rabbit, the corpus luteum is an organ of internal secretion. In these investigations extracts of corpora lutea of swine, injected into recently spayed adult

rabbits or into immature rabbits whose uteri had been brought to the mature resting state by injections of estrogen, induced alterations of the endometrium characteristic of pregnancy. These workers further demonstrated that, if female rabbits are mated and then castrated eighteen hours later while fertilized ova are still in the fallopian tube, extracts of corpora lutea substitute for the removed ovaries so completely that embryos become implanted, nourished, and maintained until full term. The presence of a functional corpus luteum in pregnancy, pseudopregnancy, and in the normal cycle in animals exhibiting a distinct luteal phase has since been demonstrated in a number of species by various experimental techniques.

The corpus luteum of ovulation in animals lacking a distinct luteal phase (hence with a short diestrual interval) commanded considerably less attention in early investigations than did the corpus luteum of pregnancy, pseudopregnancy, or the corpus luteum of animals with a distinct luteal phase. The idea that the corpus luteum of ovulation in the mouse is nonfunctional unless caused to become persistent by sterile copulation, pregnancy or lactation (Parkes and Bellerby, 1927) is typical of the earlier concepts advanced. Deansley (1938) states that the corpora lutea of ovulation in the hamster, which exhibits a four-day estrous cycle, develop rapidly after rupture of the follicle and soon become solid. He supposed that the corpus

luteum of ovulation must be functionless because the changes in the accessory organs commonly associated with the luteal phase of the cycle are found only in pseudopregnancy and pregnancy, in hamsters.

Despite lack of intensive investigation considerable physiological, histological and experimental evidence indicates progestin production in mammals prior to ovulation. The fact that heat in the guinea pig (Dempsey, et al., 1936), in the rat (Boling and Blandau, 1939), in the mouse (Ring, 1944), and in the hamster (Frank and Fraps, 1945; Kent and Liberman, 1949) is induced by the synergistic action of estrogen and progesterone suggests luteal activity and production of progestational hormone at the time of corpus luteum formation or earlier. It is notable that luteal tissue has been demonstrated in the follicles of the shrew (van der Horst and Gillman, 1940) prior to ovulation. Certain luteal-like changes have been detected in the walls of pre-ovulatory follicles in the mare (Seaborn, 1925), mouse (Allen, 1941), pocket gopher (Mossman, 1937), porcupine (Mossman, 1940), rat (Boling, et al., 1941), cat (Dawson and Friedgood, 1940), and in the bat (Wimsatt, 1944).

Evidence of an experimental nature attesting to the probability that progestin is produced prior to ovulation may be found in the experiments of Reynolds and Friedman (1930), who demonstrated a decrease in rhythmical uterine contractions characteristic of heat

between the fifth and eighth hour after copulation in the rabbit. By the time of ovulation, or even before, the uterus was completely quiescent. It was later shown (Reynolds and Allen, 1932) that injections of progesterone-containing extracts of swine corpora lutea inhibit the uterine motility previously induced by intravenous injection of theelin. Additional evidence is provided by positive Bitterling ovipositor responses (indicative of the presence of progestins) to follicular fluid of the cow and sow (Duyvene de Wit, 1938, quoted from Young, 1941). Boling, Barton and Hurr (1940) further showed that experimental injections of estrogen in spayed female rats induced steady EMF's characteristic of the beginning of heat, whereas injections of estrogen followed by progesterone induced fluctuations similar to those which appear during heat. Everett (1943) showed that single doses of 0.5-1.0 mg. of progesterone induced ovulation during the first two or three days of estrus in persistent estrus rats. Astwood (1939) observed that the water content of the rat's uterus rose to a maximum shortly before cyclic proestrus, fell abruptly with the first appearance of cornified cells in the vaginal smear, and reached a minimum at about the time of ovulation. The conclusion that this decrease in water content was the result of the secretion of progestin by the pre-ovulatory follicle was based on the experimental observation that the injection of estrogen invariably resulted in a marked

increase in the tissue fluid, and that this increase could be inhibited by progesterone injections.

With the perfection of bio-assay methods of reasonable accuracy, a new approach to hormonal determinations was made possible. Not only could the presence of a hormone be ascertained, but, in some cases, an estimate of the amount of hormone present could be made. A thorough review of the assays available for detection of the hormones of the corpus luteum is presented by Emmens (1950). Of historical significance is the Corner and Allen test for progestins (1929), using proliferation of the uterine endometrium of the estrogen-primed rabbit as a test medium. Although the assay as originally devised could not be readily adapted for the purpose of accurate measurement, nearly all research leading to the determination of progestins utilized modifications of this technique.

The observation has been made independently several times that the stromal nuclei of pregnant mice uteri are different from the nuclei of castrates. Hooker's preliminary experiments (1940, 1945) showed that the endometrium of the mouse exhibits an apparently specific nuclear response to progesterone. A systemic dose of 0.125 mg. of progesterone elicited this response; but it was later found that very small amounts of progesterone injected into the lumen of the uterus elicited the response, i.e., a concentration of 0.0002 µg.

per 0.0006 cc. of test solution (Hooker and Forbes, 1947). Specifically, the assay devised by Hooker and Forbes consists of injection, by means of microbolt action on a syringe, of 0.0006 cc. of test solution into a 5 mm. ligated segment of the uterine horn of a young adult mouse of a highly imbred strain ovariectomized sixteen days earlier. The animal is autopsied forty-eight hours after injection and the uterine horn is removed, fixed, sectioned, and stained, and the stromal nuclei observed microscopically. Under these conditions the minimal effective dose of progesterone is 0.0002 μg. The criterion for a positive response is the transformation of dense, fusiform, stromal nuclei with clumped chromatin and no distinct nucleoli, characteristic of the castrate assay animal, into plump, oval nuclei with conspicuous nucleoli and fine, evenly dispersed chromatin.

Any bio-assay of value must be characterized by specificity of response. Hooker and Forbes (1949b, 1954) selected a group of compounds which were expected to occur in the blood, those expected to be precursors or metabolites of progesterone, and those compounds having a molecular structure similar to progesterone. Thirty-three such compounds were tested. Two of these compounds duplicated the action of progesterone on the stromal nuclei. The positive response evoked by 6.144 μg. of Δ^{11}-progesterone is not of practical significance, since one-half this amount (still relatively

a huge quantity) failed to evoke a positive response. Pearlman's Compound X, isolated from the bile of pregnant cattle, evoked a positive response. Pearlman and Cerceo (1948) did not identify Compound X. The extent to which it differs from progesterone and the possible significance of the difference remain unclear. Desoxycorticosterone acetate and testosterone proprionate administered systematically in large doses duplicated the action of progesterone upon the endometrium; but when administered topically the failed to give a positive response, suggesting the conversion of a fraction of the subcutaneously injected compounds into progesterone rather than the possibility of progestational activity being an inherent property of the two compounds.

Salhanick, et al., (1951) sometimes obtained false negative responses when assaying the progesterone content of monkey and rat sera using the Hooker-Forbes technique. In these instances it was suspected that considerable amounts of estrogen were present in the samples. In order to test the possibility of a blocking action of estrogen, these workers assayed, with the Hooker-Forbes technique, graded amounts of alpha estradiol combined with constant amounts of progesterone in sesame oil and in mammalian Ringer's solution. Solutions containing estradiol and progesterone in ratios of 1:200 or proportionally more estradiol gave negative responses. It was concluded that estrogens block the action of progesterone on the stromal nuclei. Zarrow and Neher (1951,

1953) performed similar experiments. They found that estradiol blocked the reaction at a ratio of 1:20, estrone at a ratio of 1:1, and that estriol failed to block at a ratio of 60:1. The blocking action was shown in this experiment, as in that of Salhanick, et al., to depend on the ratio of estrogen to progesterone, and not on the absolute amounts of either. It should be further noted that circulating estrogen is not estradiol, but probably estriol, bound to a protein (Roberts and Szego, 1946). This estrogen is much less active than estradiol, as has been shown by many investigators, and it has no antagonistic action to progesterone at a ratio of sixty parts of estriol to one part of progesterone. Courrier (1950) has reviewed the antagonism of estrogen and progesterone and showed that it occurs in a wide variety of organs.

Reports of progesterone content of blood, serum, or plasma during the pre-ovulatory phase of the cycle are rare. Neher and Zarrow (1950, 1954) report 2.0 µg. to 0.3 µg. of progesterone per cc. of serum at estrus in the ewe. No reference was made as to whether ovulation had occurred, but these authors state that progestin was present during the entire estrous cycle and suggested its presence in the blood in the absence of the corpus luteum. These results are in accord with the findings of Forbes, Hooker and Pfieffer (1950a) and Bryans (1951) that progesterone is present in the circulating blood during the follicular phase of the menstrual cycle in monkeys, and in

accord with the work of Forbes (1950, 1951) on the human being, but not in accord with the failure to find progesterone in the blood of pregnant women (Haskins, 1950; Butt, et al., 1951) and in the peripheral blood of nonpregnant cows, mares, ewes, sows (Edgar, 1953). Fraps, Hooker and Forbes (1948) report the presence of 0.3 µg. of progesterone per cc. of plasma in the ovulating hen. Fraps, et al., (1949), using the Hooker-Forbes assay method, have shown progesterone to be present in reproductively quiescent hens and cocks in the amount of 0.33 µg. per cc. of plasma. Forbes (1948) reported the presence of 5 µg. of progesterone per cc. of plasma in the mouse at time of impregnation.

Edgar (1953), using a chemical assay, found the fluid from the mature Graafian follicle of the cow to contain 3 µg. of progesterone per cc. In the sow he found 8 µg. of progesterone per cc. of fluid. These findings are in very close agreement with the earlier works of Hooker and Forbes.

Recently, Forbes (1953), using the Hooker-Forbes assay method, determined progesterone levels of the peripheral blood of rabbits after mating, and following injections of gonadotrophins at intervals of minutes, hours, or days. Maximum progesterone levels in samples drawn during the first 3 or 4 hours were 0.3-0.8 µg. per cc. of plasma for eight of the mated animals, and 1.1-3.9 µg. per cc.

of plasma for seven injected specimens. Free progesterone appeared an average of 100 minutes after mating and 64 minutes after intravenous administration of gonadotrophins, thus providing direct evidence that the rabbit secretes progesterone prior to ovulation. Zarrow and Neher (1955) report a range of 0.3 to 1.0 µg. progestin per cc. of serum at the time of mating in the rabbit.

No data are available concerning the progesterone content of plasma during the estrous cycle of laboratory animals exhibiting a short diestrual period. Since the ultimate resolution of the questions of controls, antagonisms, and synergisms of hormones of the corpus luteum will probably result from experiments utilizing common laboratory animals, it seemed that knowledge of the plasma progestin levels of the hamster during the normal cycle would be of value. The present study was therefore undertaken with three questions in mind: (1) Is plasma progestin present during the pre-ovulatory phase of the cycle? (2) What amount of free progestin is in the plasma during estrus, early diestrus, mid-diestrus and late diestrus? (3) Is there a distinct post-ovulatory increase in progestin indicating an active luteal phase in the estrous cycle of the golden hamster?

The Hooker-Forbes assay technique has several disadvantages such as time and labor required, the manifestation of an all-or-none response which necessitates the use of a series of dilutions or

concentrations in order to pick out the sample containing the minimal effective quantity, the acquisition of a high level of skill in assessing the sections of the uterine test segment, and the inhibitory action of estrogens under certain conditions. However, in view of the high level of sensitivity and specificity, the wide variety of materials which can be tested, the low cost of test animals, and the small space required for their housing, this bio-assay technique was chosen in preference to other techniques for the determination of free progestin in the plasma of the golden hamster during the estrous cycle.

MATERIALS AND METHODS

Two hundred and eighty-three adult female mice of the Rockland Farms Swiss-albino strain were employed in the present investigation. At the time of ovariectomy the mice were 65 to 79 days of age and weighed from 22 to 30 grams. The original stock was obtained from Rockland Farms, Rockland County, New York. Additional test animals were reared. Young mice were weaned at about three and one-half weeks of age, and sister littermates were placed in separate cages. Members of the colony of assay mice (which usually included about 300 individuals), as well as the test animals, were maintained on a diet of Purina Dog Chow, oats and water supplied ad libitum.

Members of the above strain apparently exhibit no peculiarities in response or sensitivity to progesterone. Tests of sensitivity of the assay animals were made prior to the experiment, and were verified during the course of the experiment, by injecting from 3.0 μg. to 0.00006 μg. of progesterone in oil (Eli Lilly and Co.) into the mice to be tested (Table 1). Present data confirm the observations of Zarrow (q. v., page 7) and Olsen, Salhanick and Hisaw (1952) that animals from the Rockland Farms Swiss-albino strain are sensitive to 0.0002 μg. of progesterone per 0.0006 cc. of test

solution.[1] In view of the observations of Hooker and Forbes (q.v., page 6), and Zarrow and Neher (q.v., page 7), and Salhanick, et al., (q.v., page 7), no tests were conducted on the effects of related steroids. However, observations were made of the effects of castration, of injection of sesame oil alone, and of ligation of a segment without injection, on the stromal nuclei. These observations, with the exception of ligation of the segment without injection, have been suggested by the authors of the assay as prerequisite to any study utilizing the assay.

Bilateral ovariectomy of the mice was performed using a single abdominal incision under Nembutal (sodium pentobarbital) anaesthesia. The Nembutal was prepared periodically as an aqueous solution containing 20 mg. per cc. of the solution. The amount injected for anaesthesia was approximately 0.09 mg. per gram of body weight. This amount usually induced complete relaxation for about an hour. All observable effects of anaesthesia were gone by the end of three or four hours. The ovaries surrounded by considerable adjacent fat, the fallopian tubes, and 2 to 3 mm. of the adjacent

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[1] In view of the apparent discrepancies of biological and chemical findings the identity of the substances assayed by the Hooker-Forbes bio-assay is uncertain. The progesterone like activity of the plasma has been standardized against progesterone and will be referred to in this report as progestins.

uterine horns were removed. Bleeding was controlled by placing hemostats on the ovarian arteries and at the tips of the uterine horns. Even though the mouse appears to be highly resistant to bacterial infection, the area of the incision was sprinkled lightly with sulfadiazine. Separate nylon sutures were placed in the muscles and in the skin of the animal. It was unnecessary to remove subsequently the skin sutures, since the mice regularly removed them, sometimes as early as the second post-operative day. The sutures of the muscular wall of the abdomen were left in situ.

Exactly sixteen days after ovariectomy assay mice were again anaesthetized and prepared for injection. The uterine horn was delivered through a single midventral incision located posterior to the incision used for ovariectomy, and a nylon ligature was placed near the cranial end of the horn. Care was taken to exclude from the ligature the large vessels which course in the mesometrium parallel to the uterus. At least two overhand knots were tied, using light tension in each case.[2] A second ligature employing the same precautions was placed 5 mm. caudal to the first, using a single, loose, overhand knot. After making certain that the needle was filled with

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[2] Light tension was employed since segments tied tightly, as originally suggested by the authors of the assay, invariably caused swelling and negative responses.

the fluid to be injected, the horn was grasped by forceps about 3 mm. caudal to the posterior ligature, and the needle was inserted, beveled side up, into the uterine lumen immediately cranial to the forceps and pushed forward almost to the anterior ligature. The desired volume of fluid was then discharged into the horn, after which the horn was withdrawn from the needle. During withdrawal of the horn sufficient tension was maintained on the threads to produce a constricting angle in the horn. In order to maintain tension while removing the horn, three hands would have been required. Therefore, a holding device for the syringe was constructed. The use of this device made possible the maintenance of slight tension on the threads which were held with forceps during the withdrawal of the uterine horn from the needle. At least two overhand knots were again tied.

The animal was killed 48 hours after injection and the injected uterine segment was removed and fixed in Lavdowsky's fluid (William and Hodges, 1943), a fixative which, according to Hooker and Forbes (1947), causes a minimum of shrinkage. Sections were cut six microns in thickness and stained with Harris' hematoxylin.

The "Agla" micrometer syringe (Fig. 1) was developed at the Wellcome Research Laboratories, Beckenham, Kent, England, for use in experimental techniques requiring the injection of very small volumes of fluid. It consists of a calibrated, all-glass,

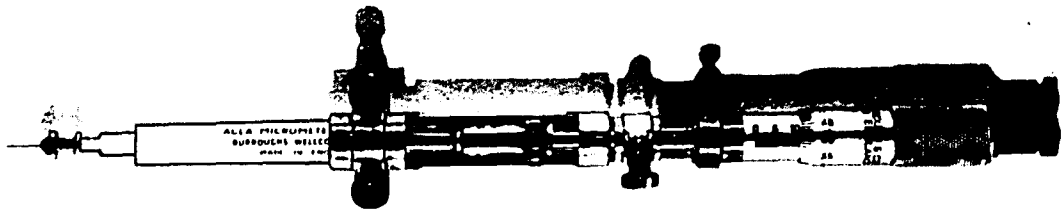

Fig. 1. "Agla" micrometer syringe and metal holder.

hypodermic syringe which is held in position by a rigid, metal holder. A micrometer screw gauge operates the plunger. This syringe is essentially similar to those used by Hooker and Forbes and other authors employing the assay. One complete revolution of the micrometer head advances the plunger 0.5 mm., delivering a volume of 0.01 ml. of fluid. The total graduated travel of the micrometer (50 revolutions of 25 mm.) thus delivers a volume of 0.5 ml. The peripheral scale of the micrometer head is divided into fiftieths of a revolution. Each unit on this scale corresponds to a volume of 0.0002 cc. By turning the micrometer head through three of the peripheral divisions, one can inject 0.0006 cc.,[3] the amount required for the bio-assay.

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[3] The two syringes used were calibrated by filling the syringe with mercury and delivering and weighing numerous measured quantities of mercury. It was found that by turning the micrometer head through three of the peripheral divisions one syringe actually delivered 0.000590 cc.; the other syringe delivered 0.000612 cc.

The eighty-six female hamsters used in this experiment were of L.S.U. stock and, for the most part, had reared one litter. Twelve per cent of the hamsters were young adult virgins. All animals were caged individually and maintained on Purina Dog Chow, oats, lettuce and water supplied ad libitum. At the time blood samples were taken no hamster used was younger than 2-1/2 months of age or older than 7-1/2 months of age. Over eighty per cent of the animals used were 4-6 months of age. They ranged in weight from 76 to 132 grams. Eighty per cent of the animals used were 80-105 grams in weight. In all cases, fresh unstained vaginal smears were observed nightly between 6 P.M. and 8 P.M. during at least two consecutive cycles prior to collection of blood for assay. Animals showing an irregularity in the estrous cycle were discarded. Animals from which estrual blood samples were drawn were also tested for mating responses; and as soon as the female exhibited psychic estrus the blood sample was collected. No estrual blood sample was drawn earlier than 6:40 P.M. or later than 10:10 P.M. (Chart 2). According to the observations of Ward (1946), these samples represent a pre-ovulatory phase in the cycle.

Since the assay method allows the use of serum, plasma, or saline dilutions thereof (Hooker and Forbes, 1947), and since the use of plasma seemed to be the most expedient method, initial tests were

conducted on pure plasma and serial dilutions (0.9% NaCl) thereof. Blood samples (usually about 3.5 cc.) were taken from the post-caval vein with a 5-cc. syringe and a 20-gauge, 1-1/2 -inch needle. The syringe contained 0.07 cc. of 40% sodium citrate as an anti-coagulant. Samples were promptly centrifuged for 30 minutes at 3000 RPM. After plasma samples were separated from the packed cells serial dilutions were prepared and assayed. These initial tests proved negative indicating a need for concentrating the progestin per unit volume as compared with the original plasma concentration.

The method used by Hooker, Forbes, Zarrow, and others to separate and concentrate the progestins is a modification of the method used by Szego and Roberts (1947) to separate protein-bound estrogens. This modification was used in the present investigation. The plasma samples obtained after centrifugation, usually 2 cc., were added drop by drop while constantly stirring to approximately 10 volumes of acetone at 3°C in order to precipitate the proteins.[4]

After having remained at 3°C overnight the solution was quickly centrifuged and decanted. The protein precipitate was

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[4] The acetone was placed in 50 cc. erlenmeyer flasks, and the temperature reduced by placing the flasks in a refrigerator at 3°C. In order to maintain this low temperature while dropping the plasma into the acetone, it was found necessary to place the erlenmeyer flasks in beakers lined with paper or bagass. This assured only minimal changes in temperature.

washed 3 times with a 1:1 mixture of cold acetone and ether. After each washing and decantation the proteins were returned to the refrigerator for recooling to approximately 3°C before further washing. The precipitate was tightly capped and not allowed to dry. At each washing the protein precipitate was resuspended. The washings were combined with the supernatant and a measured volume of sesame oil was added. The protein precipitate, which contains two-thirds of the estrogens (Szego and Roberts, 1946) and approximately ten per cent of the total progesterone (Hooker and Forbes 1949a, Edgar 1953), was discarded. The acetone and ether were then removed by distillation under diminished pressure provided by a water vacuum pump. Most of the water remained and was later separated from the oil containing the free progestin.[5] Since the remaining one-third of the estrogens are hydrophilic and are apparently of an ester type (Szego and Roberts, 1946), they are eliminated along with the water following centrifugation. It has been shown by Hooker and Forbes that samples prepared in the above described manner assay practically identically with whole blood, raw plasma, or serum,

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[5] Whether or not this fraction is truly 'free' progestin is uncertain. It is possible that all progestin is bound to a protein, most of it so loosely that the methods employed would not distinguish it from 'free' progestin. For convenience this fraction is referred to as 'free'. The fraction requiring rigorous hydrolysis to free it from the protein is referred to as 'bound' progestin.

as to free progestin content.

Using the foregoing technique it was possible to concentrate progestin per unit volume of oil as compared with the original concentration per unit volume of plasma. It was sometimes necessary to pool the acetone-ether extracts from several blood samples in order to prepare very concentrated samples. The extracts were stored at 3°C until they could be combined and prepared in the usual manner.

Distillation time varies directly proportionately to the volume to be distilled and inversely as the temperature. The time required was reduced by immersing the claisen distillation flask in a luke-warm water bath. Since the boiling point of acetone or ether is low, and extremely low under vacuum, little warmth was needed to increase the rate of distillation. Even with this technique, final traces of acetone (which are very discernible by sniffing) are removed slowly. A 40 cc. sample, one of the smallest, usually required 2 to 3 hours for complete distillation.

The original level of concentration of progestin per cubic centimeter of plasma was low; therefore, in order to prepare samples which would give positive responses, the amount of sesame oil added to any sample of acetone-ether extract was small. The losses in the 150 cc. claisen distillation flask, in the pipette used to withdraw the

oil sample therefrom, in the 3-cc. centrifuge tube used in centrifugation of the water-oil mixture, and in the micro pipette used to withdraw the oil from the surface of the water, prevented the recovery of a sizeable portion of the original volume of sesame oil added. The final sample of progestin in oil was very small. In most instances serial dilutions were feasible only with samples prepared from blood drawn during estrus.

RESULTS

Sensitivity of Response of Mouse Strain Employed

It has been shown by various workers that mice of the Swiss-albino strain (Rockland Farms) are sensitive to 0.0002 µg. of progesterone per 0.0006 cc. of sesame oil. In the present investigation a concentration of 0.0002 µg. of progesterone, or more, per 0.0006 cc. of sesame oil consistently evoked positive nuclear responses (Table 1). Of twelve uterine segments injected with 0.0002 µg. of progesterone, nine exhibited positive nuclear responses. Of the nine giving positive responses, six were injected following the standard interval of time and all six gave positive responses. This is in good agreement with the findings of Zarrow, who reports (personal communication) ninety per cent positive responses under similar circumstances. These sensitivity experiments indicate that mice of the Swiss-albino strain possess a sensitivity to progesterone comparable to that of the CHI strain used by the authors of the assay. Zarrow and Neher (1951, 1954) and Olsen, et al., (1951) reached a similar conclusion before electing to employ the Rockland-Swiss strain.

The injection of 0.0002 µg. of progesterone or more into the lumen of the uterus of the castrate mouse resulted in the enlargement

Table I. Response from assays designed to determine sensitivity of mouse strain employed.

A. Micrograms of Progesterone per 0.0006 cc.	I.D.[1]	No. of Injections or Uterine Segments	Responses[2]
3.00000	19	3	///
0.00500	19	4	////
0.00043	35	3	///
0.00043	16	8	///////-
0.00024	16	6	//////
0.00021	35	4	///-
0.00020	19	5	//---
0.00020	16	7	///////
0.00015	16	6	------
0.00006	16	6	------
B. Controls			
Ligated Segment	16	10	----------
Sesame Oil	16	10	----------
Ovariectomized	16	10	----------

1. I.D. refers to the interval between ovariectomy and injection.
2. Each / or – refers to the response of one individual test segment.

Table II. Assay responses elicited by progestin samples of varying concentrations and dilutions prepared from estrual blood.

Dilution (D) or Concentration (X)	I.D.[1]	Assay Responses[2]	Hour Sample Taken (P.M.)	Numbers of Donors[3]
15X	16	+++++	8:00-8:20	137;140;146
15X	16	+++++++-	9:20-9:40	190;192;205
12X	20	++++--	8:00-8:20	125;133;145
12X	16	++++	8:00-8:20	125;133;145
10X	16	+++++	6:40-6:50	113;116
8X	17	+++++	8:00-8:20	153;154;156
6X	16	+++++++-	7:45-8:00	127;155;158
4X	16	+++++++	7:50-8:10	106;138;159
2X	17	+++++	9:30	110
2X	16	+++-	8:50	227
1.5X	16	+++++	7:45-8:00	168;169
1.3X	16	++++-----	8:30-8:45	161;167
1X	16	++++-	9:00-9:15	164;174
1X	16	++++--	9:35	103
Plasma	16	------	10:10	219
2D	16	----	9:00-9:15	164;174
3D	16	+---	8:50	227
4D	16	------	9:00-9:15	164;174
4D	16	++++	7:50-8:10	106;138;159
5D	16	----	8:30-8:45	161;167
8D	16	------	9:00-9:15	164;174
10D	16	------	10:10	219

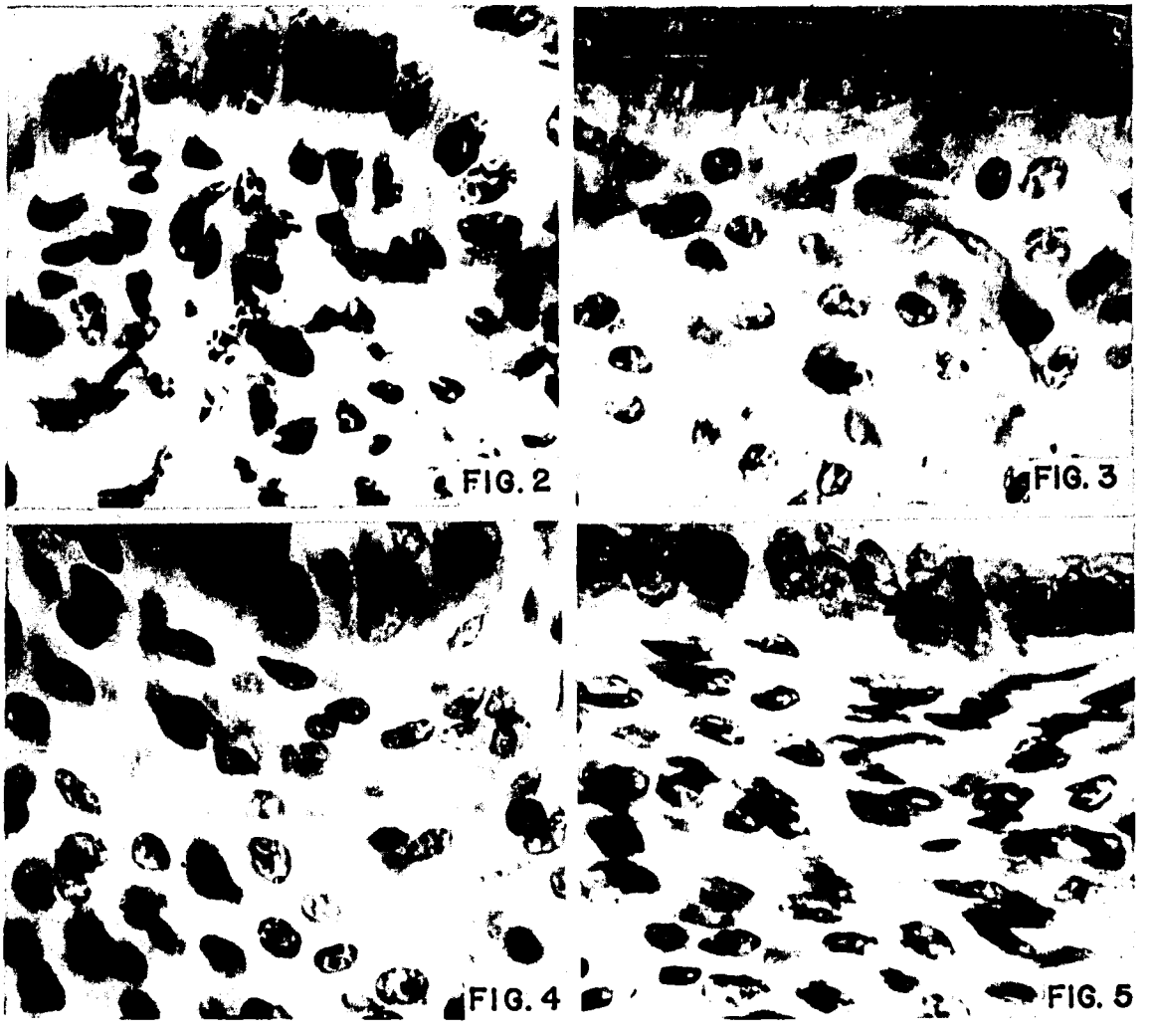
1. See explanatory note 1, Table I.
2. See explanatory note 2, Table I.
3. Blood pooled to prepare sample.

of the stromal nuclei (Figs. 3, 4), which assumed a smooth, slightly elongated, oval outline. The chromatin particles were fine and evenly distributed, and the nucleolus was conspicuous. All these conditions must be met before the response is considered positive. It is necessary to distinguish between stromal nuclei and the nuclei of the glandular epithelium. The latter nuclei are recognized as belonging to the uterine glands by examining adjacent serial sections.

Absence of Response in Control Mice

Nuclei with a shrunken fusiform appearance, with clumped chromatin, and with no recognizable nucleolus are characteristic of the castrate condition (Fig. 2). It has been shown that 16 days is the minimal interval after ovariectomy in which all the endometrial stromal nuclei of the mouse will exhibit the full castrate condition (Hooker and Forbes, 1947). All uterine segments of castrate, uninjected mice excised on the 16th post-operative day were negative (Table I).

Sesame oil alone injected under assay conditions failed to evoke positive responses in castrate mouse uteri (Table I). Although the nuclei were perhaps slightly enlarged, the characteristic positive reaction to progesterone was not evoked in any of these uteri (Fig. 5). Uterine segments from castrate mice ligated in the usual manner but without injection also failed to show positive responses (Table I).



Photomicrographs (X 1225) of typical sections of endometria of mice ovariectomized 16 days previously.

Fig. 2. No injection (16 day post-operative condition). The stromal nuclei are shrunken and have clumped chromatin.

Fig. 3. After the injection of 0.0002 μg. of progesterone into the lumen of the uterine segment. A characteristic stromal nucleus is oval and has a conspicuous nucleolus and fine, evenly dispersed chromatin particles.

Fig. 4. After the injection of 0.00043 μg. of progesterone into the lumen of the uterine segment. A characteristic stromal nucleus is likewise oval and has a conspicuous nucleolus and fine, evenly dispersed chromatin particles.

Fig. 5. After injection of sesame oil alone. The stromal nuclei are spindle-shaped and have large, irregularly spaced chromatin particles. There is no distinct nucleolus.

Free Plasma Progestin Levels During Pre-ovulatory Estrus

In the present investigation the highest concentration of progestin was detected during pre-ovulatory estrus. This is not surprising in view of the generally accepted absence of a distinct luteal phase in the estrous cycle in the hamster. All pre-ovulatory estrus samples of original concentration (1X) and above consistently elicited positive assay responses (Table II), indicating the presence of 0.3 ug. of progestin per cc. of plasma. While most samples diluted two or more times gave negative responses (Table II), one sample prepared at 4D evoked a positive response and one sample prepared at 3D gave a questionably positive response. A range of 0.3 to 1.2 µg. of progestin per cc. of plasma, therefore, was detected at estrus. In view of the negative responses elicited by samples prepared at 2D, 4D, 5D, 8D, and 10D, 0.3 ug. of progestin per cc. of plasma appears to be the highest concentration that can be regularly detected at early pre-ovulatory estrus. This range almost coincides with the range reported for the rabbit and the ewe during estrus. The peak of concentration of progestin in the blood of the rabbit is attained several hours after mating, but prior to ovulation (Forbes, 1953). This is probably true also for the hamster. A further study of progestin levels at successive intervals during the entire pre-ovulatory period should be undertaken.

The negative assay response of the one plasma sample and the one dilution thereof (TableII), requires explanation. Instead of being refrigerated at -20° C until use, the sample was kept at 3°C overnight. It has been shown that incubation of serum with an original concentration of 6 µg. of progestin per cc. for two hours at 37°C will cause a loss of 17 per cent of the activity, whereas incubation for 4 hours at 37°C will cause a loss of 97 per cent of the activity (Zarrow et al., 1954). Whether the negative response was due to the faulty technique in refrigeration of the sample which allowed inactivation of the progestin, or whether the sample merely contained less than 0.3 µg. of progestin per cc. is problematical. The latter explanation seems more likely in view of the short interval of time the sample was kept at a relatively low temperature.

Free Plasma Progestin in Diestrus

Free progestin was present in low concentrations in the plasma of the hamster 24 hours post-estrus. All samples concentrated 10 times or more consistently evoked positive responses, whereas samples prepared at less than 10 times concentration failed to elicit positive responses when assayed (Table III). The maximum progestin content detected at early diestrus was 0.03 µg. per cc. of plasma. From these results it appears that the level of free progestin in the plasma decreased during the 24 post-estrual hours to

Table III. Assay responses elicited by progestin samples of varying concentrations prepared from early diestrual blood.

Concentration (X)	I.D.[1]	Assay Responses[2]	Hour Sample Taken (P.M.)	Numbers of Donors[3]
15X	16	++++++++++ +++-	8:30-8:45	176;191;193
12X	17	+++++++	8:00-9:00	120;134;141
12X	16	++++++	8:00-9:00	120;134;141
11X	16	++--	8:25	214
10X	16	+++++---	8:55	204
10X	16	+---	9:05-9:20	162;182
9X	16	+-------	8:30-8:45	144;172
9X	16	------	9:00-9:15	157;175
8X	16	----------	8:30-8:45	149;160
6X	16	----------	9:05	221
6X	16	---------- --	8:50	199
6X	16	------	8:30-8:45	149;160
4X	16	---------- --	8:00-8:15	148;151
3X	16	----------	7:20-7:35	165;182
2X	16	----	8:00-8:15	148;151
1X	16	----	8:00-8:15	148;151

1. See explanatory note 1, Table I.
2. See explanatory note 2, Table I.
3. Blood pooled to prepare sample.

less than one-tenth the pre-ovulatory level (Fig. 6). The maximum progestin content at mid-diestrus in the animals tested was likewise 0.03 µg. per cc. of plasma. Samples concentrated 10 times or more consistently gave positive responses, whereas samples concentrated less than 10 times gave negative responses (Table IV). The amount of free progestin at mid-diestrus is thus approximately the same as the amount found during early diestrus, but less than one-tenth as much as that found in estrus. Assays on samples taken approximately 72 hours post-estrus continue to show the presence of progestin (Table V). The maximum progestin content detected during late diestrus was 0.03 µg. per cc. of plasma. This is approximately equal to the amount detected at early diestrus and mid-diestrus. There appears to be no significant rise or fall in the amount of free progestin in plasma during the diestrual phases of the cycle.

Cyclic Variations in Levels of Free Progestin

Free progestin was found to be present in varying concentrations during the entire estrous cycle. The amount of free progestin detected during various phases of the estrous cycle is presented graphically (Fig. 6). The successive progestin concentrations detected at estrus, early diestrus, mid-diestrus, and late diestrus are connected by straight lines to aid visualization, but this

Table IV. Assay responses elicited by progestin samples of varying concentrations prepared from mid-diestrual blood.

Concentration (X)	I.D.[1]	Assay Responses[2]	Hour Samples Taken (P.M.)	Numbers of Donors[3]
24X	16	+++++	8:40-8:55	224;226
20X	16	+++++++++ ++++--	7:30-7:45	187;188;189
17X	16	+++-	8:55-9:05	235;239
13X	16	+++---	7:25-7:40	233;198
10X	16	+++++---- ----	8:40-8:55	121;142;152
10X	16	------	9:22-9:34	181;185
9X	16	----	8:55-9:05	183;184
8X	16	--------	7:30-7:45	178;179
5X	17	--------	8:10-8:25	150;163
5X	16	------	8:10-8:25	150;163

1. See explanatory note 1, Table I.
2. See explanatory note 2, Table I.
3. Blood pooled to prepare sample.

Table V. Assay responses elicited by progestin samples of varying concentrations prepared from late diestrual blood.

Concentration (X)	I. D. [1]	Assay Responses[2]	Hour Sample Taken (P. M.)	Numbers of Donors[3]
25X	16	++++--	7:10-7:35	209;234
20X	16	+++++-	7:25-7:40	238;236
17X	16	+++++++++ / -	7:30-7:45	207;240
14X	16	+++++++++-	8:30-8:45	230;231
12X	16	+---	8:25	229
11X	16	+++++++++	8:40-8:55	142;171
10X	16	++------	7:50-8:05	139;143
8X	16	--------	7:40-7:55	173;177
5X	16	+--------- / --------	8:00-8:15	166;170
2X	16	----	7:50-8:05	139;143
1X	16	+---	7:50-8:05	139;143
1X	16	----	8:40-8:55	147;171

1. See explanatory note 1, Table I.
2. See explanatory note 2, Table I.
3. Blood pooled to prepare sample.

is not to imply that plots of samples taken at intermediate periods would necessarily coincide with these lines. The maximum concentration of free progestin detected during the cycle, 1.2 µg. per cc. of plasma, was found at estrus. However, if additional samples had been taken nearer the time of ovulation, the peak of progestin concentration would perhaps be between estrus and early diestrus. The concentration 24 hours post-estrus dropped to less than a tenth the concentration during estrus and was maintained at this level for approximately 48 hours (Fig. 6).

By extending the graph to include three estrous cycles, (inset Fig. 6), there is seen to be a cyclic variation in the concentration of progestin in the plasma of the hamster during the cycle.

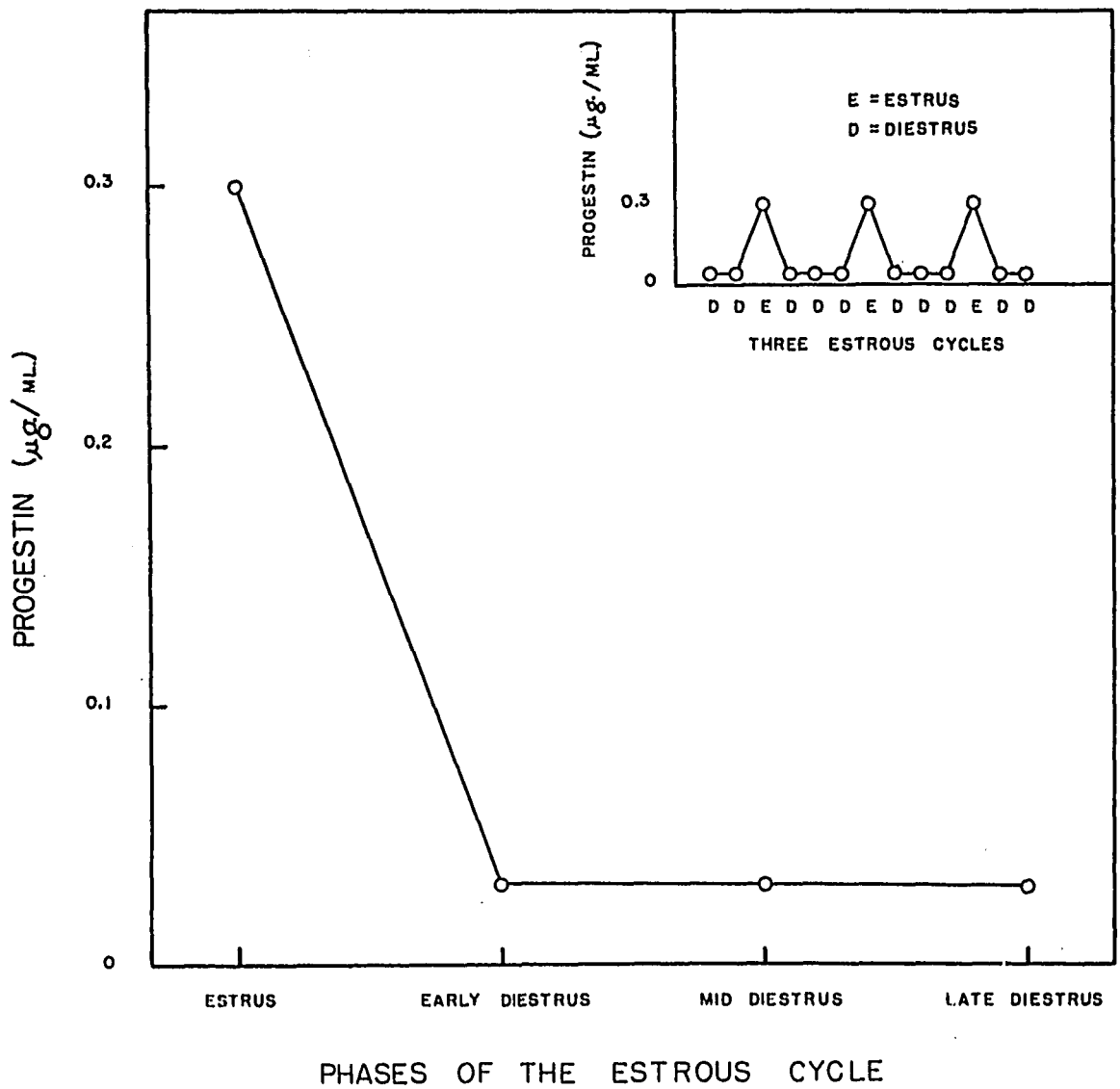


Fig. 6. Concentration of progestin in the blood plasma of the hamster during various phases of the estrous cycle. Inset: Cyclic variation of the level of progestin during three estrous cycles.

DISCUSSION

Since most chemical tests have failed to detect progesterone in the peripheral blood of pregnant women (Haskins, 1950, using ultraviolet spectroscopy; Butt, et al., 1951, using polarographic method), and in nonpregnant cows, mares, ewes and sows (Edgar, 1953, using a chemical assay); and since the Hooker-Forbes bio-assay has detected progesterone in the blood of a pregnant woman (Hooker and Forbes, 1949a), in pregnant women and monkeys (Forbes, 1951), in the blood of the menstrual cycle of the monkey (Forbes, et al., 1950a; Bryans, 1951), and in the blood of the pregnant and nonpregnant ewe (Neher and Zarrow, 1950, 1954), the question presents itself as to whether the Hooker-Forbes bio-assay should be regarded as specific for progesterone, or for progestins. Very recently Zander (1954), using physico-chemical procedures, detected progesterone in samples prepared from peripheral blood of pregnant women. It appears that the Hooker-Forbes bio-assay reliably determines progesterone when present, but may also demonstrate levels of progesterone higher than those which can be detected by other methods apparently sensitive enough to do so.

It is quite possible that there is a circulating hormone in addition to progesterone which is detected by the Hooker-Forbes

bio-assay, but which is undetected by other methods utilized. This could explain the discrepancy in biological and chemical findings. In view of this possibility, the progesterone like activity of the blood measured by the Hooker-Forbes bio-assay, and which is standardized against progesterone, should probably be expressed as progestins based on equivalents of progesterone. Such a terminology is commonly employed when expressing estrogen concentrations in blood.

In view of the foregoing considerations, one cannot contend that the data presented herein are quantitatively exact. On the other hand, it seems reasonably certain that they do indicate the nature of the general fluctuations in concentration of progestins in the plasma during the estrous cycle.

The presence of progestin in amounts of the magnitude of 0.3 µg. per cc. of plasma during the pre-ovulatory phase of the cycle is not surprising, as considerable evidence for its appearance previous to ovulation has been gathered for several species of mammals (summarized on pages 3 and 4). There is relatively little anatomical evidence of such pre-ovulatory secretion, however. It may be that cells scattered in the walls of the follicle assume a luteal function. Changes have been reported in the walls of unruptured follicles in the late pre-ovulatory stage (summarized on page 3).

No detailed histological description of the growth and rupture of the follicle in the hamster has been found in the literature.

According to Deansley (1938), the corpora lutea of ovulation of the hamster develop very rapidly, and generally disappear before the end of the following cycle. He further states that the corpora lutea must be functionless, since the changes in the accessory organs associated with the luteal phase of the cycle are found only in pseudopregnancy and pregnancy. The presence of 0.03 µg. of progestin per cc. plasma during early diestrus, mid-diestrus, and late diestrus, as determined in the present investigation, poses a question concerning the source of this progestin, and concerning the threshold amount of progestin necessary to evoke progestational changes in the reproductive tract. However, in view of the work of Zarrow, et al., (1954), who report a 90 per cent decrement of intravenously injected progesterone within ten minutes, and of Haskins (1950), who reports a 60 per cent loss of intravenously injected progesterone in thirty minutes, it is difficult to reconcile even 0.03 µg. of progestin per cc. of plasma at 48 to 72 hours following a production period limited to near the time of ovulation. On the other hand, the detection of progesterone in the blood of a vein draining one ovary of a pregnant ewe containing two mature Graafian follicles and no corpus luteum, and the failure to find progesterone in the general circulation of that animal (Edgar, 1953), suggests the secretion of progesterone by well-developed Graafian follicles

present during pregnancy. Two mature follicles of the ewe produced enough progesterone to give a concentration of 1 µg. of progesterone per cc. of blood leaving that ovary. Could six to fourteen follicles, less mature, produce enough progestin to give a concentration of 0.03 ug. progestin per cc. plasma in the hamster?

Whether the corpus luteum of ovulation is functional is still problematical. The presence of approximately 0.03 µg. of progestin per cc. of plasma during the entire diestrual phase of the cycle is easily explainable on the assumption that the corpus luteum of ovulation is producing a small quantity of progestin. This amount could be assumed to be below the threshold concentration necessary to maintain a progestational condition in the accessory reproductive organs.

Production of progesterone by the adrenal glands was demonstrated by Beall and Reichstein (1938), when they extracted progesterone from the adrenals of oxen. A review of the experiments indicating production of progesterone by the adrenals has been presented by Burrows (1950). Recently, it has been shown that concentrations of progestins comparable to the 0.03 µg. of progestin per cc. of plasma herein found during the diestrual phases of the hamster cycle are found in the male, and this progestin has been identified as coming from the adrenal gland (Lazo-Wasem, et al., 1954). The

adrenal gland has been suggested likewise as the source of progestin during the puerperium of the rabbit (Zarrow and Neher, 1955). The general absence of progestin in the blood of castrated animals and in normal animals during menstruation indicates that an extra-ovarian source does not contribute appreciably to the total circulating progestin. Nevertheless, the adrenals cannot be excluded as a possible source for the small amount of progestin detected during the diestrual phase of the cycle in the hamster, nor can the adrenals be excluded as a source of a portion of the amount detected at estrus.

Comparison of the amounts of progestin present in the blood during estrus in various species reveals that the concentrations may vary considerably. The highest concentration reported thus far is 5 ug. of progesterone per cc. of plasma at the time of impregnation in the mouse (Forbes, 1948). Zarrow and Neher (1955) reported at the time of mating in the rabbit a range of 0.3 to 1.0 µg. of progestin per cc. of serum. Neher and Zarrow (1950, 1954) reported a range of 0.3 to 2 µg. of progestin per cc. of serum in the ewe. In the hamster at estrus, as herein reported, 0.3 to 1.2 µg. of progestin per cc. of plasma has been detected. It is quite possible that, if samples had been taken nearer the time of ovulation, slightly higher concentrations of progestin would have been found. Why the concentration in the mouse should be twice as high as the concentration in

other forms tested is problematic. Could the presence of three clearly discernible sets of corpora lutea in the spontaneously ovulating mouse (Allen, 1922) be responsible for the higher level of progestin secretion? The exhibition in the mouse of three sets of corpora lutea at estrus is in contrast to the condition in the hamster, in which there is no accumulation of corpora lutea.

SUMMARY

1. Free progestin levels in the blood plasma of the golden hamster were determined at various phases of the estrous cycle by the Hooker-Forbes bio-assay.
2. The data presented are based on the assay responses of 540 uterine test segments.
3. Eighty-eight normal, cyclic hamsters served as blood donors.
4. Assays were conducted on oil samples prepared from acetone-ether extracts of blood drawn during pre-ovulatory estrus, early diestrus, mid-diestrus, and late diestrus.
5. Free progestin was present in detectable quantities throughout the estrous cycle. Concentrations of 0.3 to 1.2 μg. of progestin per cc. of plasma were detected at pre-ovulatory estrus. During the 24 post-estrual hours this level dropped to 0.03 μg. of progestin per cc. of plasma. A concentration of 0.03 μg. of progestin per cc. of plasma was detected during mid-diestrus and late diestrus.
6. If the corpus luteum of the diestrual interval is functional, it is certainly functional at a low level of activity.
7. The source of the plasma progestin present during pre-ovulatory estrus and during the diestrual phases of the cycle requires further investigation.

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VITA

William Warren Norris, Jr., was born at Choudrant, on March 2, 1927. He received his elementary and secondary education in the public schools of Lincoln Parish. He was graduated from Choudrant in 1944 and entered Louisiana Polytechnic Institute, Ruston, in July of that year. He entered the Army of the United States in May, 1945, served in the European Theater of Operations and received an honorable discharge in October, 1946. In February, 1947, he reentered Louisiana Polytechnic Institute which conferred upon him the degree of Bachelor of Science in January, 1950. He then entered the Graduate School of Louisiana State University in the Department of Zoology in February, 1950. While at L.S.U. he served as Graduate Assistant in the Department of Zoology during the school years 1951 to 1955. In August, 1951, the degree of Master of Science in Zoology was conferred upon him. He continued in advance study and research to become a candidate for the degree of Doctor of Philosophy in the Department of Zoology in the Louisiana State University and Agricultural and Mechanical College. On December 23, 1949, he married Frances L. Freeman of Ruston, Louisiana. They have two sons, Dennis Stephen and Joel William Norris.

EXAMINATION AND THESIS REPORT

Candidate: Mr. William Warren Norris, Jr.

Major Field: Zoology

Title of Thesis: "Free Progestin Levels in the Blood Plasma of Hamsters during the Estrous Cycle."

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

July 23, 1955